

TABLE OF CONTENTS

	Page Number
APPENDICES.....	x
LIST OF FIGURES	xiv
LIST OF TABLES.....	xvi
LIST OF ACRONYMS AND ABBREVIATIONS	xx
ACKNOWLEDGEMENTS	xxiii
PREFACE.....	xxvi
EXECUTIVE SUMMARY.....	xxxi
1.0 INTRODUCTION AND RATIONALE FOR THE USE OF <i>IN VITRO</i> NEUTRAL RED UPTAKE CYTOTOXICITY TEST METHODS TO PREDICT STARTING DOSES FOR <i>IN VIVO</i> ACUTE ORAL SYSTEMIC TOXICITY TESTING	1-3
1.1 Background and Rationale for the Use of <i>In Vitro</i> Cytotoxicity Assays to Predict Starting Doses for <i>In Vivo</i> Acute Oral Systemic Toxicity Tests	1-6
1.1.1 The MEIC Program	1-6
1.1.2 The RC	1-8
1.1.3 The ZEBET Initiative to Reduce Animal Use	1-11
1.1.4 The NICEATM/ECVAM <i>In Vitro</i> NRU Cytotoxicity Validation Study	1-12
1.2 Regulatory Rationale and Applicability for the Use of <i>In Vitro</i> Cytotoxicity Test Methods to Predict Starting Doses for Acute Oral Systemic Toxicity Testing	1-15
1.2.1 Current Regulatory Testing Requirements for Acute Systemic Toxicity	1-15
1.2.2 Intended Regulatory Uses for the <i>In Vitro</i> Cytotoxicity Test Methods	1-18
1.2.3 Similarities and Differences in the Endpoints of the <i>In Vitro</i> Cytotoxicity Test Methods and <i>In Vivo</i> Acute Oral Toxicity Test Methods	1-18
1.2.4 Use of <i>In Vitro</i> Cytotoxicity Test Methods in the Overall Strategy of Hazard Assessment	1-20
1.3 Scientific Basis for the <i>In Vitro</i> NRU Test Methods.....	1-21

1.3.1	Purpose and Mechanistic Basis of the <i>In Vitro</i> NRU Test Methods	1-22
1.3.2	Similarities and Differences in the Modes/Mechanisms of Action for the <i>In Vitro</i> NRU Test Methods Compared with the Species of Interest.....	1-23
1.3.3	Range of Substances Amenable to the <i>In Vitro</i> NRU Test Methods	1-23
2.0	TEST METHOD PROTOCOL COMPONENTS OF THE 3T3 AND NHK <i>IN VITRO</i> NRU TEST METHODS.....	2-3
2.1	Overview of the 3T3 and NHK NRU Test Methods.....	2-4
2.1.1	The 3T3 NRU Test Method	2-7
2.1.2	The NHK NRU Test Method	2-8
2.1.3	Measurement of NRU for both 3T3 and NHK Test Methods.....	2-9
2.2	Descriptions and Rationales of the 3T3 and NHK NRU Test Methods	2-9
2.2.1	Materials, Equipment, and Supplies.....	2-9
2.2.2	Reference Substance Concentrations/Dose Selection	2-12
2.2.3	NRU Endpoints Measured	2-14
2.2.4	Duration of Reference Substance Exposure.....	2-15
2.2.5	Known Limits of Use	2-16
2.2.6	Nature of Response Assessed.....	2-17
2.2.7	Appropriate Vehicle, Positive, and Negative Controls.....	2-18
2.2.8	Acceptable Ranges of Control Responses	2-19
2.2.9	Nature of Experimental Data Collected.....	2-20
2.2.10	Type of Media for Data Storage.....	2-21
2.2.11	Measures of Variability	2-21
2.2.12	Methods for Analyzing NRU Data.....	2-22
2.2.13	Decision Criteria for Classification of Reference Substances	2-23
2.2.14	Information and Data Included in the Test Report.....	2-23
2.3	Basis for Selection of the <i>In Vitro</i> NRU Cytotoxicity Test Methods.....	2-25
2.3.1	<i>Guidance Document</i> Rationale for Selection of <i>In Vitro</i> NRU Cytotoxicity Test Methods.....	2-25
2.3.2	<i>Guidance Document</i> Rationale for Selection of Cell Types.....	2-26
2.4	Proprietary Components of the 3T3 and NHK NRU Test Methods	2-28

2.5	Basis for Number of Replicate and Repeat Experiments for the 3T3 and NHK NRU Test Methods.....	2-28
2.6	Basis for Modifications to the 3T3 and NHK NRU Test Method Protocols	2-29
2.6.1	Phase Ia: Laboratory Evaluation Phase	2-29
2.6.2	Phase Ib: Laboratory Evaluation Phase	2-33
2.6.3	Phase II: Laboratory Qualification Phase	2-37
2.6.4	Phase III: Laboratory Testing Phase.....	2-44
2.7	Differences in 3T3 and NHK NRU Test Method Protocols and the <i>Guidance Document</i> Standard Protocols.....	2-45
2.7.1	Optimization of the <i>Guidance Document</i> Protocols Prior to Initiation of the Study	2-45
2.7.2	Optimization of the <i>Guidance Document</i> Protocols During the Study	2-47
2.8	Overview of the Solubility Protocol.....	2-48
2.9	Components of the Solubility Protocol.....	2-50
2.9.1	Medium, Supplies, and Equipment Required	2-50
2.9.2	Data Collection.....	2-51
2.9.3	Variability in Solubility Measurement	2-52
2.9.4	Solubility and the 3T3 and NHK NRU Test Methods.....	2-52
2.9.5	Methods for Analyzing Solubility Data.....	2-52
2.10	Basis of the Solubility Protocol.....	2-53
2.10.1	Initial Solubility Protocol Development.....	2-53
2.10.2	Basis for Modification of the Phase II Protocol.....	2-54
2.11	Summary	2-55
3.0	REFERENCE SUBSTANCES USED FOR VALIDATION OF THE 3T3 AND NHK NRU TEST METHODS	3-3
3.1	Rationale for the Reference Substances Selected for Testing.....	3-3
3.1.1	Reference Substance Selection Criteria.....	3-3
3.1.2	Candidate Reference Substances.....	3-5
3.1.3	Selection of Reference Substances for Testing	3-15
3.2	Rationale for the Number of Reference Substances Selected	3-16
3.3	Characteristics of the Selected Reference Substances	3-17
3.3.1	Source Databases Represented by the Selected Reference Substances	3-17

3.3.2	Chemical Classes Represented by the Selected Reference Substances	3-21
3.3.3	Product/Use Classes Represented by the Selected Reference Substances	3-22
3.3.4	Toxicological Characteristics of the Selected Reference Substances	3-22
3.3.5	Selection of Reference Substances for Testing in Validation Study Phases Ib and II	3-27
3.3.6	Unsuitable and Challenging Reference Substances	3-29
3.4	Reference Substance Procurement, Coding, and Distribution.....	3-30
3.5	Reference Substances Recommended by the <i>Guidance Document (ICCVAM 2001b)</i>.....	3-32
3.6	Summary	3-32
4.0	IN VIVO RODENT TOXICITY REFERENCE VALUES USED TO ASSESS THE ACCURACY OF THE 3T3 AND NHK NRU TEST METHODS	4-3
4.1	Methods Used to Determine <i>In Vivo</i> Rodent Toxicity Reference Values	4-3
4.1.1	Identification of Candidate <i>In Vivo</i> Rodent Toxicity Reference Data	4-3
4.1.2	Criteria Used to Select Candidate <i>In Vivo</i> Rodent Toxicity Data for Determination of Reference Values	4-6
4.2	Final <i>In Vivo</i> Rodent Toxicity Reference Values	4-10
4.3	Relevant Toxicity Information for Humans.....	4-16
4.4	Accuracy and Reliability of the <i>In Vivo</i> Rodent Toxicity Reference Values	4-16
4.5	Summary	4-20
5.0	3T3 AND NHK NRU TEST METHOD DATA AND RESULTS	5-3
5.1	3T3 and NHK NRU Test Method Protocols.....	5-3
5.1.1	Phase Ia: Laboratory Evaluation Phase	5-4
5.1.2	Phase Ib: Laboratory Evaluation Phase	5-5
5.1.3	Phase II: Laboratory Qualification Phase	5-6
5.1.4	Phase III: Main Validation Phase	5-7
5.2	Data Obtained to Evaluate Accuracy and Reliability.....	5-9
5.2.1	PC Data	5-10
5.2.2	Reference Substance Data.....	5-12

5.3	Statistical Approaches to the Evaluation of 3T3 and NHK NRU Data.....	5-12
5.3.1	Statistical Analyses for Phase Ia	5-13
5.3.2	Statistical Analyses for Phase Ib	5-15
5.3.3	Statistical Analyses for Phase II.....	5-15
5.3.4	Statistical Analyses for Phase III.....	5-16
5.4	Summary of Results	5-18
5.5	Coded Reference Substances and GLP Guidelines.....	5-36
5.5.1	Coded Reference Substances	5-36
5.5.2	Lot-to-Lot Consistency of Reference Substances	5-36
5.5.3	Adherence to GLP Guidelines	5-36
5.6	Study Timeline and NICEATM/ECVAM Study Participatory Laboratories	5-37
5.6.1	Statement of Work and Protocols.....	5-37
5.6.2	Study Timeline	5-38
5.6.3	Participatory Laboratories.....	5-38
5.7	Availability of Data	5-39
5.8	Solubility Test Results.....	5-39
5.8.1	Solubility Data.....	5-40
5.8.2	Solubility Effects on the <i>In Vitro</i> NRU Cytotoxicity Test Method Data.....	5-45
5.9	Summary	5-47
6.0	ACCURACY OF THE 3T3 AND NHK NRU TEST METHODS	6-3
6.1	Accuracy of the 3T3 and NHK NRU Test Methods for Predicting Acute Oral Systemic Toxicity.....	6-5
6.1.1	Linear Regression Analyses for the Prediction of <i>In Vivo</i> Rodent LD ₅₀ Values from <i>In Vitro</i> NRU IC ₅₀ Values	6-5
6.1.2	Comparison of Combined-Laboratory 3T3 and NHK NRU Regressions to the RC Millimole Regression	6-6
6.2	Improving the Prediction of <i>In Vivo</i> Rodent LD₅₀ Values from <i>In Vitro</i> NRU IC₅₀ Data.....	6-8
6.2.1	The RC Rat-Only Regression in Millimolar Units.....	6-9
6.2.2	The RC Rat-Only Regression in Weight Units	6-10
6.2.3	The RC Rat-Only Regression in Weight Units Excluding Substances with Specific Mechanisms of Toxicity	6-12

6.3	Accuracy of the 3T3 and NHK NRU Test Methods for Toxicity Category Predictions.....	6-14
6.3.1	Prediction of Toxicity Category by the 3T3 and NHK NRU Test Methods Using the RC Millimole Regression.....	6-17
6.3.2	Prediction of Toxicity Category by the 3T3 and NHK NRU Test Methods Using the RC Rat-Only Weight Regression	6-22
6.3.3	Prediction of Toxicity Category by the 3T3 and NHK NRU Test Methods Using the RC Rat-Only Weight Regression Excluding Substances with Specific Mechanisms of Toxicity	6-26
6.3.4	Summary of the Regressions Evaluated	6-30
6.4	Strengths and Limitations of the 3T3 and NHK NRU Test Methods for <i>In Vivo</i> Toxicity Prediction	6-31
6.5	Salient Issues of Data Interpretation.....	6-35
6.6	Comparison to Established Performance Standards.....	6-36
6.7	Summary	6-41
7.0	RELIABILITY OF THE 3T3 AND NHK NRU TEST METHODS.....	7-3
7.1	Substances Used to Determine the Reliability of the 3T3 and NHK NRU Test Methods.....	7-4
7.2	Reproducibility Analyses for the 3T3 and NHK NRU Test Methods	7-6
7.2.1	ANOVA Results for the 3T3 and NHK NRU Test Methods.....	7-16
7.2.2	CV Results for the 3T3 and NHK NRU Test Methods	7-17
7.2.3	Comparison of Laboratory-Specific Linear Regression Analyses for the Prediction of <i>In Vivo</i> Rodent LD ₅₀ Values from <i>In Vitro</i> NRU IC ₅₀ Values.....	7-24
7.2.4	Laboratory Concordance for the Prediction of GHS Acute Oral Toxicity Category.....	7-24
7.3	Historical Positive Control Data.....	7-27
7.3.1	ANOVA and Linear Regression Results for the 3T3 NRU Test Method	7-30
7.3.2	ANOVA and Linear Regression Results for the NHK NRU Test Method	7-32
7.4	Laboratory Concordance for Solvent Selection.....	7-34
7.5	Summary	7-37
8.0	3T3 AND NHK NRU TEST METHOD DATA QUALITY	8-3
8.1	Adherence to Good Laboratory Practice Guidelines.....	8-3
8.1.1	Guidelines Followed for <i>In Vitro</i> NRU Cytotoxicity Testing.....	8-3

8.1.2	Quality Assurance (QA) for <i>In Vitro</i> NRU Cytotoxicity Test Data.....	8-5
8.1.3	Guidelines Followed for <i>In Vivo</i> Rodent Oral LD ₅₀ Data Collection.....	8-7
8.2	Results of Data Quality Audits	8-8
8.2.1	QA Statements.....	8-8
8.2.2	QA Statements from the Laboratories	8-9
8.3	Impact of Deviations from GLPs/Non-compliance	8-12
8.3.1	Laboratory Error Rates	8-12
8.3.2	Test Failure Rates for Definitive Tests and PC Tests.....	8-13
8.3.3	Intralaboratory Reproducibility.....	8-15
8.3.4	Globally Harmonized System Toxicity Category Predictions.....	8-15
8.4	Availability of Laboratory Notebooks.....	8-16
8.5	Summary	8-17
9.0	OTHER SCIENTIFIC REPORTS AND REVIEWS OF <i>IN VITRO</i> CYTOTOXICITY TEST METHODS AND THE ABILITY OF THESE TEST METHODS TO PREDICT ACUTE SYSTEMIC TOXICITY	9-3
9.1	Relevant Studies	9-3
9.1.1	Correlation of <i>In Vitro</i> NRU Cytotoxicity Results with Rodent Lethality.....	9-3
9.1.2	Use of Cytotoxicity Data to Reduce the Use of Animals in Acute Toxicity Testing	9-6
9.1.3	Other Evaluations of 3T3 or NHK NRU Methods.....	9-11
9.2	Independent Scientific Reviews	9-14
9.2.1	Use of <i>In Vitro</i> Cytotoxicity Data for Estimation of Starting Doses for Acute Oral Toxicity Testing	9-15
9.2.2	Validation of 3T3 NRU for Phototoxicity	9-19
9.3	Studies Using <i>In Vitro</i> Cytotoxicity Test Methods with Established Performance Standards	9-20
9.3.1	<i>Guidance Document</i> (ICCVAM 2001b).....	9-21
9.3.2	King and Jones (2003)	9-21
9.3.3	A-Cute-Tox Project: Optimization and Pre-Validation of an <i>In Vitro</i> Test Strategy for Predicting Human Acute Toxicity (Clemedson 2005)	9-22
9.4	Summary	9-23

10.0 ANIMAL WELFARE CONSIDERATIONS (REFINEMENT, REDUCTION, AND REPLACEMENT).....	10-3
10.1 Use of 3T3 and NHK NRU Test Methods to Predict Starting Doses for Acute Systemic Toxicity Assays.....	10-4
10.2 Reduction and Refinement of Animal Use for the UDP	10-5
10.2.1 Procedure for <i>In Vivo</i> Testing Using the UDP.....	10-5
10.2.2 Procedure for Computer Simulation Modeling of the UDP	10-7
10.2.3 Animal Savings for the UDP When Using 3T3 and NHK NRU-Based Starting Doses.....	10-9
10.2.3.1 <i>The Effect of Dose-Response Slope on Animal Use.....</i>	10-9
10.2.3.2 <i>Mean Animal Use from UDP Simulations for Testing the NICEATM/ECVAM Reference Substances – Comparison of Regressions and 3T3 and NHK NRU Test Methods ...</i>	10-11
10.2.3.3 <i>Animal Savings for the UDP by Toxicity Category Using 3T3 and NHK NRU-Based Starting Doses</i>	10-13
10.2.4 Refinement of Animal Use for the UDP When Using 3T3 and NHK NRU-Based Starting Doses	10-23
10.3 Reduction and Refinement of Animal Use for the ATC	10-25
10.3.1 Procedure for <i>In Vivo</i> Testing Using the ATC.....	10-25
10.3.2 Procedure for Computer Simulation Modeling of the ATC	10-26
10.3.3 Animal Savings for the ATC When Using 3T3 and NHK NRU-Based Starting Doses.....	10-28
10.3.3.1 <i>The Effect of Dose-Response Slope on Animal Use.....</i>	10-28
10.3.3.2 <i>Mean Animal Use for ATC Simulations of Testing the NICEATM/ECVAM Reference Substances – Comparison of Regressions and 3T3 and NHK NRU Test Methods ...</i>	10-29
10.3.3.3 <i>Animal Savings for the ATC by Toxicity Category Using 3T3 and NHK NRU-Based Starting Doses</i>	10-29
10.3.4 Refinement of Animal Use for the ATC When Using 3T3 and NHK NRU-Based Starting Doses	10-40
10.4 Summary	10-41
11.0 PRACTICAL CONSIDERATIONS	11-3
11.1 Transferability of the 3T3 and NHK NRU Test Methods	11-3
11.1.1 Facilities and Major Fixed Equipment	11-4
11.1.2 Availability of Other Necessary Equipment and Supplies	11-6
11.2 3T3 and NHK NRU Test Method Training Considerations.....	11-8

11.2.1	Required Training and Expertise Needed	11-8
11.2.2	Training Requirements to Demonstrate Proficiency	11-10
11.3	Test Method Cost Considerations	11-11
11.3.1	3T3 and NHK NRU Test Methods.....	11-11
11.3.2	<i>In Vivo</i> Rodent Acute Oral Toxicity Testing	11-12
11.4	Time Considerations for the 3T3 and NHK NRU Test Methods	11-14
11.5	Summary	11-15
12.0	REFERENCES	12-1
13.0	GLOSSARY	13-1